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Biological and histopathological interactions of Korean cruciferous vegetables and *Heterodera trifolii*

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Biological and histopathological interactions of Korean cruciferous vegetables and *Heterodera trifolii*

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ABSTRACT

Biological and histopathological interactions of Korean cruciferous vegetables and *Heterodera trifolii*

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Heterodera trifolii was isolated from highland Chinese cabbage fields in Gangwon province, Korea in 2018 at 7 years after the similar cyst nematode *H. schachtii* in symptoms and morphology had been firstly reported in the same places in 2011, causing severe disease in Chinese cabbage. This study aimed to examine biological and histopathological interactions of cruciferous vegetables and *H. trifolii* to provide basic information useful for the management of the cyst nematode. In the screening of Chinese cabbage and radish cultivars for reproduction, cyst formation (number of cysts per plant) and fecundity (number of eggs per cyst) of *H. trifolii* at 28 days after inoculation (DAI) were significantly higher in Chinese cabbage than radish cultivars, indicating the nematode multiplication rates were significantly higher in Chinese cabbage than radish. Among these cultivars, Geumjeong was revealed to be resistant to the nematode as nematode population was decreased with negative multiplication rate of 0.2%, contrary to other cultivars with positive multiplication rates of 4.9–59.8%. Nematode

penetration rates increased progressively from 2 until 10 DAI to reach penetration rates in the higher order of Chinese cabbage cv. CR, radish cv. Geumjeong and Songbaek, but initial nematode growth was similar among the cultivars examined. For histopathological characteristics, syncytial areas at 10 and 15 DAI differed in relation to the nematode reproduction on the cultivars examined, but rates of syncytial areas relative to stele areas were not in the higher order of the nematode reproduction, suggesting plant damages caused by the nematode infection may not be completely coincided with the nematode reproduction. The same biological characteristics mentioned above were examined using the selected cultivars by the soil amendment with a fertilizer of increased concentrations at intervals of 0.01% (0.00–0.04%). In this study, the nematode multiplication rates were significantly higher at 0.00% and lowest at 0.02% at which plant growth were significantly higher than the other concentrations. Initial penetration rates and nematode growths differed little among the fertilizer concentrations except for 0.03% and 0.04% which inhibited greatly plant growths. All of these aspects may provide information useful for the development of the nematode control strategies.

Keywords: *Heterodera trifolii*, Chinese cabbage, radish, fertilizer

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INTRODUCTION

In Korea, Gangwon province accounts for 16.4% and 11.6% in the production of Chinese cabbage and radish with cultivation areas of 21.1% and 16.4%, respectively, in which most of the highland Chinese cabbage and radish with high productivity and quality are grown (KOSIS, 2017). However, severe damages of the highland Chinese cabbage from a cyst nematode were observed and the nematode was firstly identified as *Heterodera schachtii* in 2011, of which the nematode distribution areas were 11.6 ha in 2011 and increased to 156.6 ha in 2017 at the time of which *H. trifolii* was newly identified and caused damages the highland Chinese cabbage and radish alone and together with *H. schachtii* (Kwon *et al*., 2016; Okki *et al*., 2018).

Cyst nematodes, *Heterodera* and *Globodera* species are economically problematic nematode worldwide and have been actively studied for biological and pathological interactions with host plants (Bernard *et al*., 2017). In addition, *Heterodera* spp. infecting cruciferous vegetables are principal pests banned to be introduced into Korea by the quarantine regulations (APQA, 2019).

Heterodera species are sedentary endo-parasitic nematodes, which on syncytia formed in the feeding site of the root tissues to acquire nutrients and therefore, the nematode growth and development are closely associated with syncytial development in the feeding site (Acedo *et al*., 1984; Kim *et al*., 1987). Syncytia also protect the nematode in plant tissues from external environments (Vanholme *et al*., 2004). *H. schachtii*, pathogenic to cruciferous

crops as *H. trifolii*, has been reported to inhibit plant growth most greatly with higher inoculum population at 20℃ in the cultivation field (Kabir *et al*., 2018). Females of cyst nematode remain in the soil as the cysts that protect eggs and juveniles existing inside, thereby increasing possibilities of the nematode survival in soil and spreading to other fields during cultural practicing like plowing (Kwon *et al*., 2016).

There are three major control methods including cultural control such as crop rotation, chemical control and use of resistant plants. As a nematode control method, crop rotation to inhibit reproduction of cyst nematode can be managed to reduce the nematode population below the economic damage level (Hill, 1988); however, it is not practical for the cyst nematodes that is very persistent in soils unfavorable for the survival of the cyst nematodes because of leathery strong cyst walls efficient for protecting eggs and juveniles existing inside cysts. Chemical control using nematicides is also impractical due to low control value because of the reasons mentioned above. Also, most nematicides are highly toxic to humans and animals and environmentally harmful, so that their uses have been banned and the number of nematicides available in commercial markets has been continuously decreased.

On the other hand, use of resistant cultivars is a feasible control method of cyst nematode because of their biological characteristics as follows. Cyst nematodes are sedentary endo-parasitic nematodes that stay in plant tissues for so long period of time that the infecting nematodes are exposed to resistance responses of plants for a long period of time. Generally, cyst

nematodes have narrow host ranges, and thus there are high probabilities in success for development of resistant host plants. Also, in many cases resistance mechanisms against the cyst nematodes are expressed after infection (Kim *et al.*, 1987) that the resistant plants inhibit no or little nematode emergence from cysts and egg-hatching like non-host plants used for rotation. Besides these three control methods, it was found that the amendment of soil with organic matter; such as compost and manure to enhance crop growths was also effective in nematode control because of ammonia released from the organic matter (Hill, 1988).

Thus, this study aimed to investigate biological and histopathological characteristics in the interrelations of several cucurbitaceous vegetables and *H. trifolii* with an objective for the development of the resistance cultivars. Also, effects of fertilizer concentrations on biological characteristics of the plant-nematode interactions were examined to be applied for the nematode control and plant growth promotion as well.

MATERIALS AND METHODS

1. Nematode, plants and nematode inoculation

A nematode isolate identified as *Heterodera trifolii* by ITS gene sequencing analysis (Okki *et al*., 2018) was provided from Rural Development Administration and cultured in the Chinese cabbage cv. Koryeogeumdongi (Koryeo) for 40 days after inoculation (DAI). Five cruciferous vegetable cultivars were selected for the inoculation test, including 2 Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) cultivars CRsingsing (CR) and Koryeo and 3 radish (*Raphanus raphanistrum* subsp. *sativa*) cultivars Sokseongdaehyeong (Sokseong), Geumjeongdaehyeong (Geumjeong) and Songbaek. Seeds of the crops were purchased from commercial markets, and planted in bed soil (composed of 74.84% coco-peat, 15% vermiculite, 5% biotite, 5% pearlite, 0.158% fertilizer and 0.002% wetting agent) in 50 cell-plug trays and grown at 25℃ for seven days (until the three-true leaf seedling stages). The seedlings were transplanted in 9 cm (diameter) x 8 cm (depth) ceramic pots filled with sand soil as used in a previous study (Kim *et al*., 2017). For the inoculation test, cysts were obtained from the nematode population multiplied in Koryeo for forty days, from which nematode eggs were isolated by pressing the cysts to burst with a tweezers. Second stage juveniles (J2) were hatched from the eggs for 3–7 days by the modified Baermann funnel method, which were diluted to make a nematode suspension at the concentration of about 100 J2 / ml in sterile distilled water (SDW). Ten milliliter of the nematode suspension containing about 1,000 J2 were injected around the rhizospheres of the

Chinese cabbage and radish cultivars at two days after transplanting. The seedlings with the nematode were arranged randomly in plant growth chamber (HB-301LP, Hanbaek, Korea) and grown at 20 ± 5℃ and RH 70%, watering to the field capacity every two days during the experimentation.

2. Screening of the Chinese cabbage and radish cultivars for the reproduction of *Heterodera trifolii*

At 28 DAI, plants and soils in the pots were dumped in tap water with the plant roots macerated using hands and sieved through 20-mesh and 60-mesh sieves. The remaining soils and root debris on the 60-mesh sieve were collected in rectangular plastic plates and the number of cysts (cyst formation) was examined under a stereomicroscope (Olympus SZ-ST, Tokyo, Japan) with three replications for each cultivar. Also, the number of eggs in a cyst (fecundity) from randomly selected cysts was investigated under the stereomicroscope. The eggs collected from the cysts as mentioned above were – hatched by the modified Baermann funnel method for ten days at 25℃. The reproduction (multiplication) rates {Pf (final J2 populations) / Pi (initial J2 populations)} were measured as follows; Pf = cyst formation (no. of cysts / plant) x fecundity (no. of eggs / cyst) x hatching rate (no. of J2 / egg) and Pi = no. of nematode inoculum (1,000 J2) at the time of inoculation.

3. Screening of the Chinese cabbage and radish cultivars for the penetration and growth of *H. trifolii*

Nematode penetration and growth were examined by staining the nematodes in the roots infected with *H. trifolii*, using McCormick red food color (McCormick, Hunt valley, Maryland, USA) by the Red food color staining method (Thies *et al*., 2002; Kim *et al.,* 2017). For this, the whole roots of Chinese cabbage and radish cultivars infected with *H. trifolii* were gently pulled out from the pots at 2, 5, 10, 15 and 20 DAI, washed free of adhering soils with tap water, bleached in 1% sodium hypochlorite, and stained with 33.3% McCormick red food color (Kim *et al.*, 2017). The whole root systems were observed under the stereomicroscope to examine the number of nematodes at DAI for the nematode penetration rates compared to the initial inoculum (1,000 J2) and changes of nematode sizes and shapes among DAI for the nematode growth each with three replications.

4. Histopathological responses of the Chinese cabbage and radish cultivars to the infection of *H. trifolii*

At 10 DAI and 15 DAI, inoculated plant roots were cut 1–2cm from the root system and fixed with a modified Karnovsky's fixative {1% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2)} in a glass vial and place in a low vacuum to increase the infiltration of fixative and to remove air from root tissues. After fixation, the roots fragments were washed with 0.05 M cacodylate buffer three times for 20 minutes each and post-fixed with 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2) for 2 hours. Then the roots were washed with SDW briefly at room temperature, and *en bloc* stained with 0.5% uranyl acetate overnight at 4℃ in a refrigerator. These root segments were dehydrated in a series of ethanol (30%, 50%, 70%, 80%, 95% and three times 100%) for 10 minutes each, and further dehydrated in propylene oxide twice for 15 minutes each. These dehydrated root segments were in filtrated with the mixture of propylene oxide and Spurr's resin (1:1) followed by 100% Spurr's resin, and then polymerized at 70℃ for 8 hours in a dry oven (Spurr, 1969). The obtained resin blocks were trimmed and sectioned 1.0 μm in thickness using a diamond knife (Diatome; MF1758, Switzerland) on a MT-X ultra-microtome (RMC, Tucson, Arizona, USA). The sections were mounted on slide glass coated with albumin and stained with 1% toluidine blue in ethanol and dried on a warm plate. These sections were cleaned in a series of ethanol – mixture of ethanol and xylene (1:1) – xylene and mounted on the slide glasses with Canada

balsam, and observed under a compound light microscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

5. Effects of fertilizer concentrations on the biological relationships of cruciferous vegetables and *H. trifolii*

The fertilizer used in the experiment was Eco-sol® (Farm-Hannong, Korea) containing 25% nitrogen (N), 9% phosphorus (P) and 18% potassium (K). The fertilizer was mixed with sterilized sand soil to be diluted to 0.01%, 0.02%, 0.03% and 0.04% concentrations and no-fertilizer control (0.00%) at transplanting time of the seedlings which were inoculated with *H. trifolii* J2 as mentioned above. At 28 DAI, the plant growths (root length and shoot length) were compared among the fertilizer concentration and between the plants inoculated with and without the nematode, together with the nematode reproductions including cyst formation, fecundity, hatching rates and multiplication rates as mentioned above. Also, effects of the fertilizer concentrations on nematode penetration and growth were examined at 5, 10 and 15 DAI as mentioned above.

6. Statistical analysis

The results of reproduction, penetration of nematodes and histopathological response (syncytium formation) were analyzed statistically using one-way analysis of variance (ANOVA). Significant difference test was performed using Fisher's least significant different (LSD) test at the significant level of $P \leq 0.05$ referring to T distribution table.

RESULTS

1. Reproduction of *Heterodera trifolii* in Korean cruciferous vegetables

At 28 days after inoculation (DAI), the cyst formation (CY; no. of cysts / plant) and fecundity (F; no. of eggs / cyst) were significantly higher in the Chinese cabbage cv. CR (CY = 203.0 and F = 117.8) and Koryeo (CY = 171.7 and F = 145.5) than radish cultivars (CY = 28.7–125.3 and F = 15.0–93.5) (Table 1). Also the reproduction (multiplication) rates were significantly higher in cv. CR (x 52.2) and Koryeo (x 59.8) than the radish cultivars (x 0.2–20.9) among which radish cv. Geumjeong was revealed to be resistant to *H. trifolii* because of the nematode population reduction with negative reproduction rate of x 0.2, contrary to the population increases in other cultivars with positive reproduction rates of x 4.9–59.8. However, the hatching rate in cv. Geumjeong was not significantly different among the other cultivars except radish cv. Sokseong with the significantly highest hatching rate than the other cultivars examined in this study (Table 1).

Table 1. Reproduction of *Heterodera trifolii** in Korean cruciferous vegetables at 28 DAI

Cultivar		No. of cysts / plant	No. of eggs / cyst	Hatching rate	Pf / Pi
Chinese cabbage	CR	203.0 ± 38.6a	117.8 ± 7.6ab	2.3 ± 1.2b	52.2 ± 23.3a
	Koryeo	171.7 ± 30.0ab	145.5 ± 20.9a	2.5 ± 1.3b	59.8 ± 27.0a
Radish	Sokseong	38.3 ± 2.5c	93.5 ± 32.3bc	6.9 ± 5.0a	20.9 ± 7.6b
	Geumjeong	28.7 ± 34.1c	15.0 ± 4.1d	1.6 ± 1.7bc	0.2 ± 0.2c
	Songbaek	125.3 ± 22.9b	67.7 ± 23.7c	0.7 ± 0.5c	4.9 ± 3.8c

* About 1,000 J2 was inoculated on each plant cultivar.

Means are presented as averages ± standard deviations of three replications.

Means followed by the same letters in each row are not significantly different at $P \leq 0.05$ by LSD.

2. Penetration and growth of *H. trifolii* in Korean cruciferous vegetables

At 2, 5 and 10 DAI, the number of nematodes penetrated into the roots of three selected cultivars (cv. CR, Geumjeong and Songbaek) was examined by red food color staining method (Fig. 1). The nematode penetration rates somewhat varied depending on the time of DAI; *i.e.* in the higher order of cv. Geumjeong–Songbaek–CR at 2 DAI and cv. CR–Geumjeong–Songbaek at 5 and 10 DAI, showing penetration rates were significantly higher in Chinses cabbage cv. CR and radish cv. Geumjeong than radish cv. Songbaek (Fig. 1), which was not completely coincided with the reproduction rate that was in the higher order of cv. CR–Songbaek–Geumjeong (Table 1). The nematodes infecting the root tissues grew progressively with the increased DAI, from thread-like shapes at 2 and 5 DAI through sausage shapes at 10 and 15 DAI, and sac-like shapes at 20 DAI in all the cultivars examined; there were no significant differences in the nematode morphologies but somewhat bigger in cv. CR and Geumjeong than cv. Songbaek with the middle shapes of sausage and sac-like, compared to all sac-like shapes in cv. CR and Geumjeong (Fig. 2).

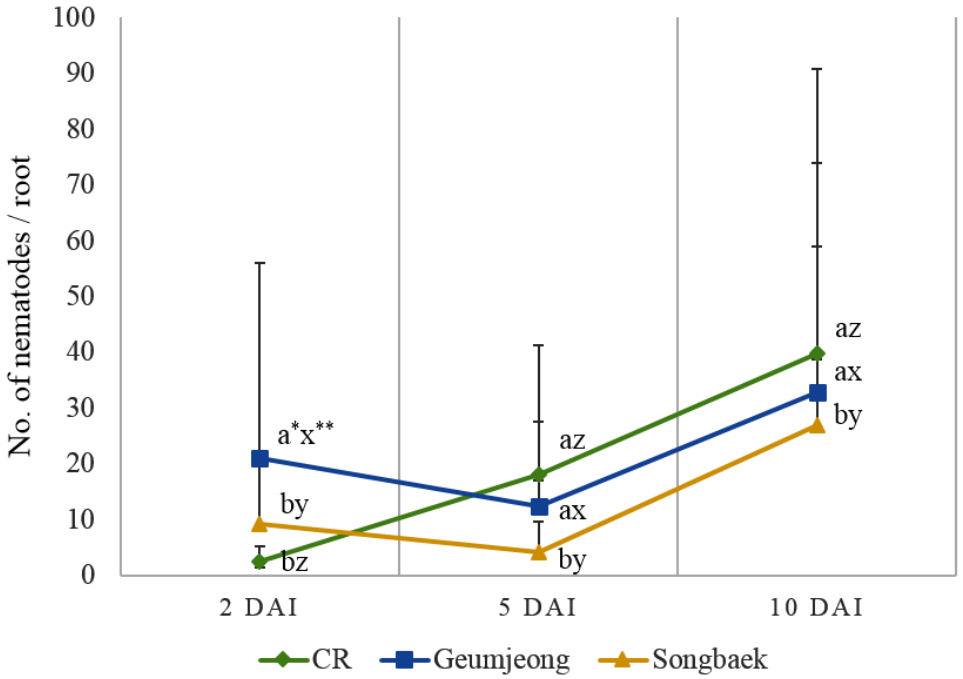

Figure 1. Penetration rates of *Heterodera trifolii* on Korean cruciferous vegetables at 2, 5 and 10 DAI (* and **; means followed by the same letters denoted no significant difference at $P \leq 0.05$ by LSD)

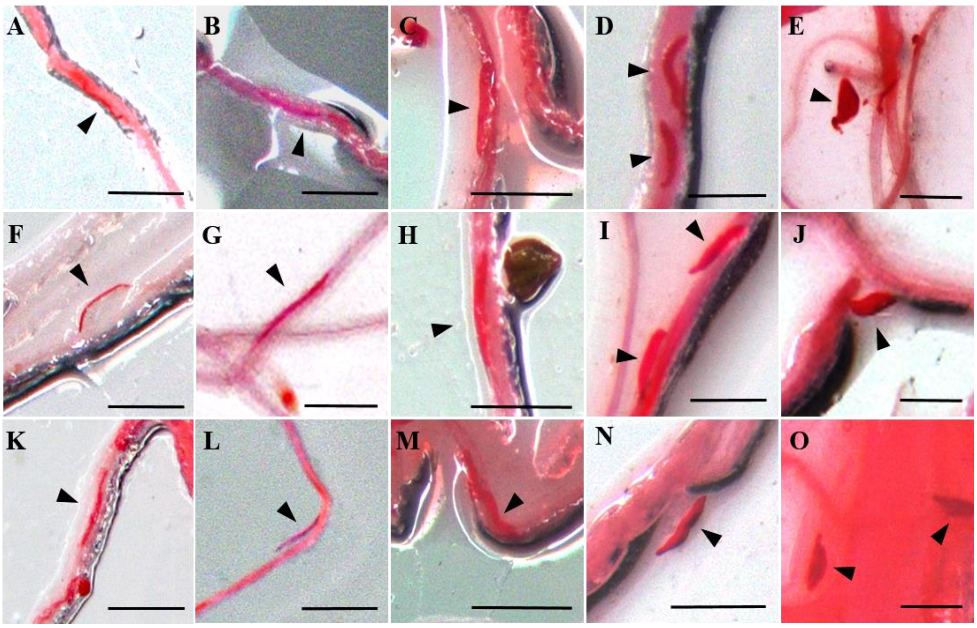

Figure 2. Light micrographs of *H. trifolii* from initial to later growths in infected cruciferous vegetable root tissues; cv. CR (A–E), Geumjeong (F–J) and Songbaek (K–O). initial growth: **{**2 DAI (A, F, K), 5 DAI (B, G, L) and 10 DAI (C, H, M)**},** later growth: {15 DAI (D, I, N) and 20 DAI (E, J, O)}. Scale bars = 500 μm

3. Histopathological responses of the cruciferous vegetables to *H. trifolii* infection

At 10 and 15 DAI, when *H. trifolii* was different in penetration and growth rates, syncytia were formed in all the cultivar roots examined, characterized by enlarged and amalgamated cells with dense cytoplasm (Fig. 3). Close examinations of the syncytial structures revealed to syncytial cell cytoplasm was highly vacuolated and indication of syncytial degeneration in cv. Geumjeong at 10 DAI. The syncytial sizes varied depending on DAI and cultivars examined; they were significantly larger in cv. CR than the other radish cultivars at 10 DAI, but cv. CR and Songbaek than cv. Geumjeong at 15 DAI (Table 2, Fig. 4). The ratios of syncytial sizes relative to stele sizes were not coincided with the syncytial sized *per se*, showing the ratios were in the higher order of cv. Songbaek, Geumjeong and CR at 10 DAI, and in the higher order of cv. CR, Songbaek and Geumjeong at 15 DAI, which were not coincided with the syncytial sized *per se* at the same time of DAI (Table 2).

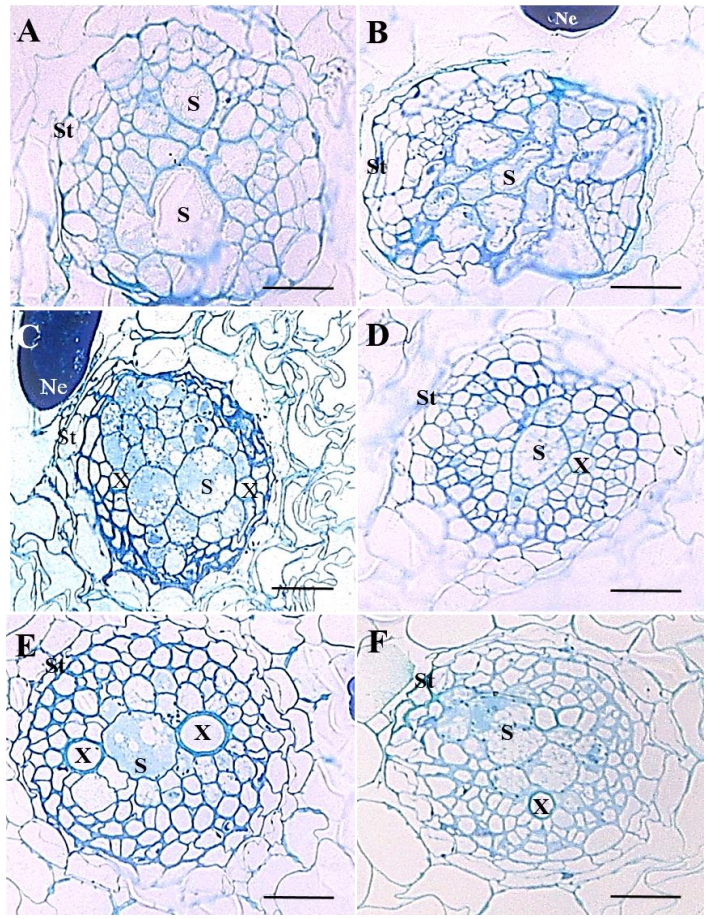

Figure 3. Light micrographs showing histopathological responses of Korean cruciferous vegetables cv. CR (A, B), Geumjeong (C, D) and Songbaek (E, F) to the infection of *H. trifolii* at 10 DAI (A, C, E) and 15 DAI (B, D, F). S: syncytium, Ne: nematode, St: stele, X: xylem vessel. Scale bars = 25 μm

Table 2. Area of the stele, syncytia and their ratio of syncytial relative to the stele in cruciferous vegetables infected with *H. trifolii* at 10 DAI and 15 DAI

DAI	Cultivar	Area (1,000 μm^2)		Ratio (%)
		Stele	Syncytia	Syncytia / Stele
10 DAI	CR	8.60 ± 0.55a	3.40 ± 0.55a	39.44 ± 4.87b
	Geumjeong	3.00 ± 1.87b	1.42 ± 1.05b	48.27 ± 17.65a
	Songbaek	0.02 ± 0.00c	0.01 ± 0.00c	50.90 ± 9.52a
15 DAI	CR	7.00 ± 0.00a	2.80 ± 0.84a	40.00 ± 11.95a
	Geumjeong	4.00 ± 0.00b	0.73 ± 0.18b	18.33 ± 4.63c
	Songbaek	6.60 ± 0.55a	1.76 ± 0.53a	27.06 ± 9.02b

Means are presented as averages ± standard deviations of five replications.

Means followed by the same letters in a column for the same DAI are not significantly different at $P \leq 0.05$ by LSD

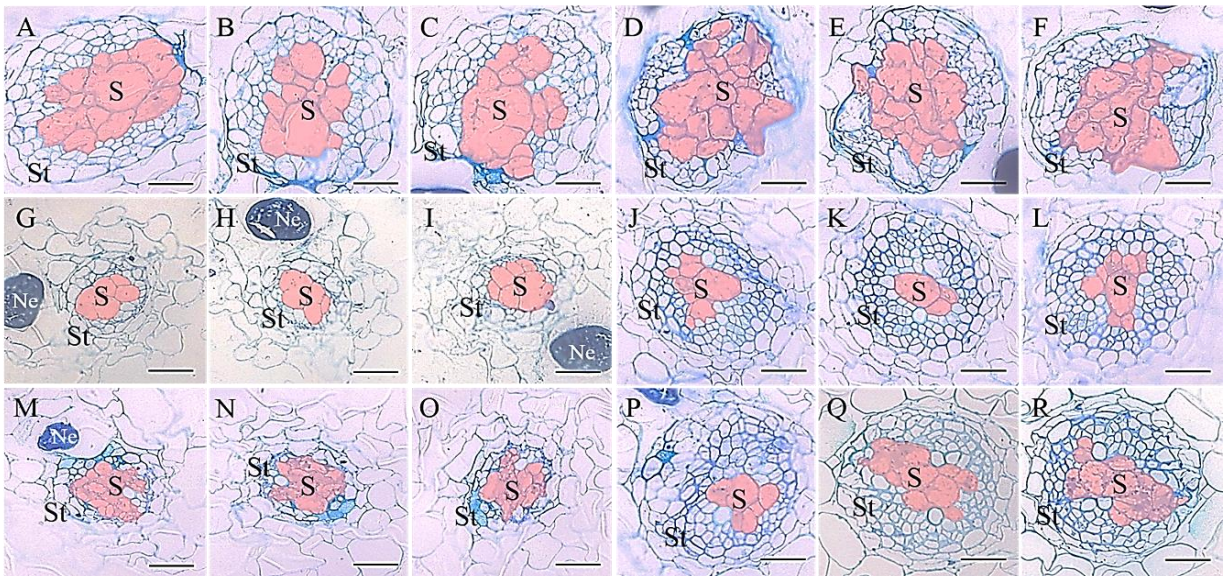

Figure 4. Light micrographs showing syncytial areas in cv. CR (A–F), Geumjeong (G–L) and Songbaek (M–R) infected with *H. trifolii* at 10 DAI (A–C, G–I, M–O) and 15 DAI (D–F, J–L, P–R). S: syncytium, Ne: nematode, St: stele. Scale bars = 25 μm

4. Effects of fertilizer concentrations on biological relationships of cruciferous vegetables and *H. trifolii*

The physico-chemical characteristics of the soil amended with different concentrations of the fertilizer were similar one another except the contents of total nitrogen (T-N), phosphate (P_2O_5) and potassium (K), showing the increased contents in soil with the increase of fertilizer concentrations applied (Table 3).

Plant growths varied depending on growth parameters (root length and shoot length), fertilizer concentrations and nematode inoculation. In non-treated control with no fertilizer, plant growths including root length and shoot length were not governed by fertilizer concentrations except for 0.02% showing significantly higher (maximum) root (19.0 cm) and shoot lengths (9.0 cm) and 0.04% showing significantly lower (minimum) root (9.5 cm) and shoot lengths (6.0 cm) (Table 4).

In the nematode inoculation, the root lengths increase linearly with increased fertilizer concentrations from 0.00% until 0.02% but decreased thereafter, showing the minimal root length of 8.67 cm at non-treated control (0.00%), maximum root length of 18.0 cm at 0.02%. On the other hand, shoot lengths were significantly shorter at 0.00% than the other fertilizer concentrations among which there were no significant differences in shoot length (Table 4). Significantly different plant growths were found at low fertilizer concentrations between inoculation and non-inoculation of the nematode; for root growth at no fertilizer treatment (0.00%) and for shoot growth at 0.01%.

For the nematode reproduction, all reproduction parameters differed significantly among the fertilizer concentrations; cyst formation with maximum at non-treated control (0.00%) and minimum at 0.02%, fecundity with maximum at 0.04% and minimum at 0.02%, with significantly higher hatching rates at 0.04%, 0.00% and 0.03%, than 0.01% and 0.02%, resulting in significantly higher reproduction rates at 0.00% and 0.04% due to highest cyst formation and fecundity, respectively, and for both due to significantly higher hatching rates than the other fertilizer concentrations (Table 5).

For the effect of fertilizer concentrations on nematode penetration and growth at DAI, the penetration rates divided into three classes; the highest at 0.00% and 0.01%, the medium at 0.02% and the lowest at 0.03% and 0.04% at which the penetrated nematodes were scarcely viewed by light microscopy (Fig. 5). The nematode growths determined by the light microscopy showed as in Figure 2; thread-like at 5 DAI, sausage shaped at 10 DAI and sac-like at 15 DAI (Fig. 6), indicating no or little effects of fertilizer applications on the penetration and growth of *H. trifolii* in Chinese cabbage cv. CR.

Table 3. Physico-chemical properties of the soil amended with different concentration of the fertilizer

Fertilizer concentration	Soil texture	pH	EC (dS / m)	OM (%)	T-N (%)	P_2O_5 (mg / kg)	K (mg / kg)	Ca (mg / kg)	Mg (mg / kg)
0.00%	Sand soil	7.42 ± 0.01	0.01	0.10	0.017	85.92	3834.54	4166.80	4528.85
0.01%	Sand soil	7.56 ± 0.03	0.01	0.11	0.016	84.42	3929.13	3976.84	4513.68
0.02%	Sand soil	7.50 ± 0.10	0.01	0.12	0.019	95.83	3942.32	3901.85	4537.54
0.03%	Sand soil	7.43 ± 0.05	0.01	0.13	0.020	92.94	3927.73	4170.96	4514.84
0.04%	Sand soil	7.22 ± 0.21	0.01	0.09	0.022	95.95	4017.53	4071.47	4520.26

The following soil characteristics were determined: organic matter by the method of Walkley & Black (1934); concentration of total nitrogen by the Kjeldahl method (Bremner, 1960); available phosphorus by the method of Bray No. 1 (1945).

Table 4. Plant growth of Chinese cabbage cv. CR amended with different concentrations of the fertilizer with and without the nematode inoculation

Fertilizer concentration	Inoculation	Plant growth	
		Root length (cm)	Shoot length (cm)
0.00%	Inoculate	8.67 ± 4.51c*	6.17 ± 2.02b
	Control	15.25 ± 1.06xy	7.75 ± 1.06xy
0.01%	Inoculate	17.00 ± 5.00a	7.67 ± 0.58a*
	Control	13.5 ± 3.53y	9.50 ± 0.00x
0.02%	Inoculate	18.00 ± 3.00a	7.67 ± 1.75a
	Control	19.00 ± 2.83x	9.00 ± 1.41x
0.03%	Inoculate	12.67 ± 3.78b	7.50 ± 1.32a
	Control	13.00 ± 5.66y	6.25 ± 1.06y
0.04%	Inoculate	11.67 ± 5.20b	7.50 ± 1.80a
	Control	9.50 ± 0.71z	6.00 ± 0.00y

Means are presented as averages ± standard deviations of three replications.

Means followed by the same letters in row for the same plant growth are not significantly different at $P \leq 0.05$ by LSD.

LSD test for all averages between inoculate and control (*, significantly different at $P \leq 0.05$).

Table 5. Effects of fertilizer concentrations on reproduction of *H. trifolii** at 28 DAI

Fertilizer concentration	No. of cysts / plant	No. of eggs / cyst	Hatching rate	Pf / Pi
0.00%	59 ± 62.01a	71.40 ± 52.24b	25.80 ± 17.71ab	1530.8 ± 1881.8a
0.01%	44 ± 41.58b	54.40 ± 28.58c	11.10 ± 7.14c	488.4 ± 541.8bc
0.02%	25 ± 31.00d	38.40 ± 27.67d	8.40 ± 6.00c	210.0 ± 298.7c
0.03%	33 ± 52.83c	50.60 ± 45.32c	20.30 ± 15.46b	670.9 ± 1204.1b
0.04%	38 ± 17.78c	105.30 ± 55.41a	35.20 ± 58.88a	1326.9 ± 2351.6a

* About 1,000 J2 was inoculated on each plant with fertilizer concentration.

Means are presented as averages ± standard deviations of three replications.

Means within a column followed by the same letters are not significantly different according to student's T test at $P \leq 0.05$.

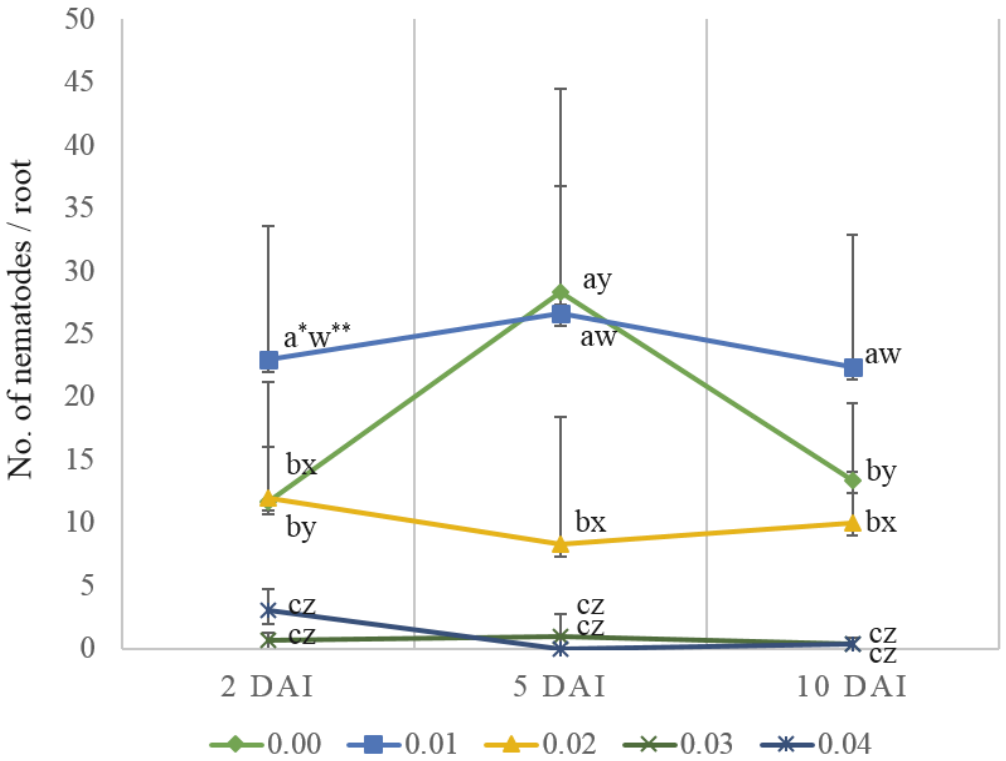

Figure 5. Penetration rates of *H. trifolii* in cv. CR treated with different fertilizer concentrations at 2, 5 and 10 DAI (* and **; means followed by the same letters in each column and row, respectively, are no significantly different at the 5% level by LSD)

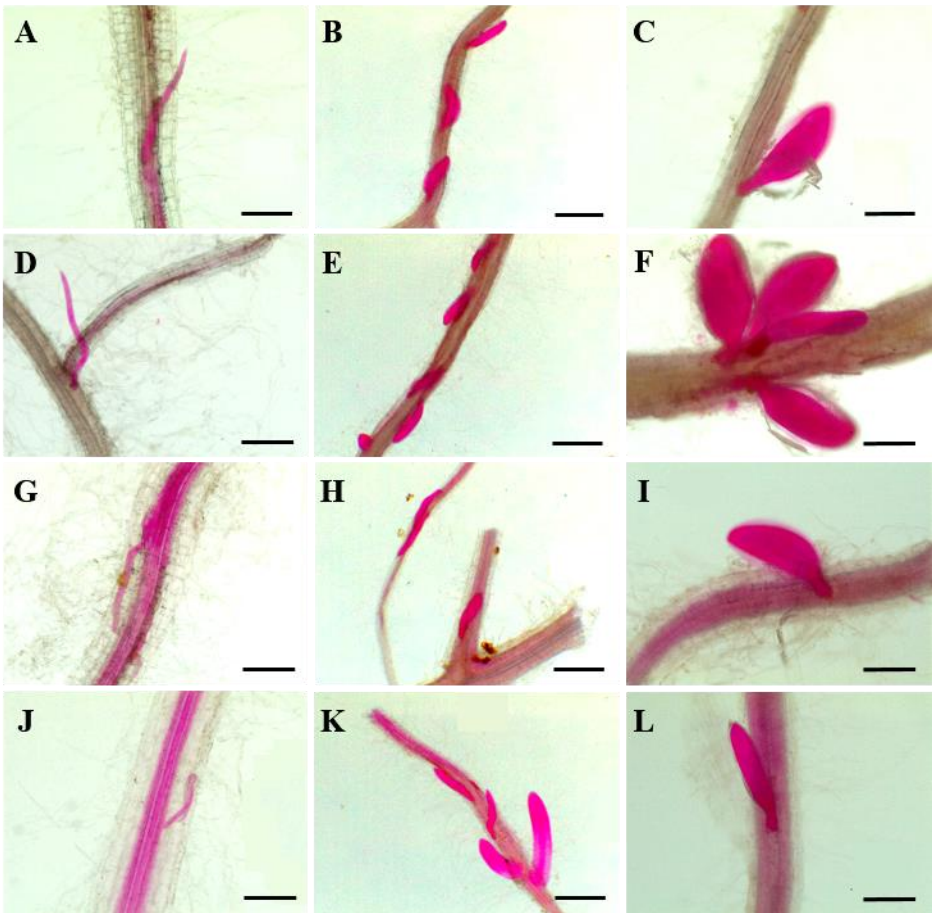

Figure 6. Light micrographs showing nematode growths in cv. CR treated with different fertilizer concentrations at 5 DAI (A, D, G, J), 10 DAI (B, E, H, K) and 15 DAI (C, F, I, L). A–C; 0.00%, D–F; 0.01%, G–I; 0.02%, J–L; 0.03%). Scale bars = 500 μm

DISCUSSION

When *Heterodera trifolii* was inoculated on 2 Chinese cabbage cultivars (CR and Koryeo) and 3 radish cultivars (Sokseong, Geumjeong and Songbaek), the cyst formation (no. of cysts / plant) of *H. trifolii* was significantly higher in the Chinese cabbage cultivars than radish cultivars although the initial penetration rates (at 2 DAI) were higher in radish cultivars (Geumjeong and Songbaek) than cv. CR. Also, there was no significant difference in the initial growth of the nematode after penetration (2, 5 and 10 DAI), but the later nematode growth (reproduction) differed in the higher order of CR, Songbaek and Geumjeong, which was consistent with syncytial size at 15 DAI (Table 1, 2). This may be due to syncytial sizes at the later stages of infection (Kim *et al*., 1999). The formation of the more enlarged syncytia influences on the nematode feeding for the growth and development (Perry and Gaur, 1996). Also feeding on syncytia provides the energy for the growth and fecundity of cyst nematodes (Van Haren *et al*., 1994).

Infective nematode population changes are governed by the nematode reproduction (multiplication) rates that are calculated by the final infective second stage juveniles (J2) population Pf {cyst formation (no. of cysts / plant) x fecundity (no. of eggs / cyst) x hatching rate (no. of J2 / egg)} / Pi {initial nematode inoculum (1,000 J2)}. Reproduction rates were also higher in the Chinses cabbage cultivars than radish cultivars, among which radish cv. Geumjeong was revealed to be resistance because of the nematode population decrease with the reproduction rate of x 0.2, which were contrary to other

cultivars which the nematode populations increased with the reproduction rates of x 4.9–59.8. These population changes were more dependent on cyst formation than other reproduction parameters such as fecundity and hatching rates in the present study because fecundity and hatching rate may offset each other to make zero-sum due to limited total nutritional supply for healthy egg formation, which may be associated with infective J2 population (fecundity x hatching rate). However, there were no significant differences in syncytial sizes between radish cv. Geumjeong and cv. Songbaek that were evaluated to be resistant and susceptible to *H. trifolii*, respectively, suggesting there may be syncytial characteristics other than the syncytial size. This may be the syncytial degeneration, indicated by highly depleted (or degenerated) cytoplasm that was seen in cv. Geumjeong at 10 DAI (Fig. 3) as syncytial degeneration is one of the resistance mechanisms to a cyst nematode (Kim *et al*., 1987). The reason for the above is that the nematode reproduction was more dependent on the formation of syncytia that provide nutritional substances for the nematode growth at the later stages of the nematode infections for reproduction (Kim *et al.*, 1999; Perry and Gaur, 1996). These aspects were also shown by the relationships of nematode reproduction, syncytial sizes and characteristics in the present study.

However, the ratios of syncytial relative to stelar sizes were not coincides with the nematode reproduction as the syncytial / stelar areal ratios were higher in radish cultivars Geumjeong and Songbaek than Chinese cabbage cultivar CR at 10 DAI (Table 2) at the time when plants are at young ages before they have adult plant resistance (Riaz *et al*., 2016). It is suggested that

extensive replacement of the vascular tissues in the stele by the syncytia is to displace vascular tissues extensively so that the longitudinal metabolite transport in susceptible hosts largely dysfunctions to cause physiological disorder of the plants infected with a cyst nematode (Kim *et al.*, 1986). This is related plant tolerance to the nematode, which is independent of plant resistance (Trudgill, 1991; Whalen, 2005).

The nematode penetration rate varied depending on the cultivars DAI; *i.e.* the significantly lower penetration rate in cv. CR than the other two radish cultivars at 2 DAI, but significantly higher in cv. CR and radish cv. Geumjeong than radish cv. Songbaek at 5 and 10 DAI, indicating penetration rate in cv. CR was significantly increased from 2 DAI to 10 DAI, while in the radish cultivars the penetration rates fluctuated with no significant changes with increased DAI (Fig. 1). The penetration was not coincided with the nematode reproduction, which can be supported by various studies that related syncytial formation to the cyst nematode reproduction (Kim *et al*., 1986, 1987, 1999). This was also supported by the fact that the nematode penetration rate was lower in radish cv. Songbaek that radish cv. Geumjeong that was significantly lower in cyst formation that cv. Songbaek (Table 1). Basically, there are no significant relationships of nematode penetration to the total expression of resistance to cyst nematode (Acedo *et al*., 1984), and the gene-for-gene relations of host-parasite interactions depend on the reproduction of the cyst nematodes, but not on the nematode penetration and establishment at the initial stages of cyst nematode infection (Kim *et al*., 1987). Resistance to cyst nematode occurs in the subsequent reaction after the feeding site in

determined, so it is likely to be after establishment of the nematode parasitism when syncytium begins to develop around the stylet followed by the enlargement of nuclei and nucleoli, formation of dense cytoplasm and cell wall alteration (Davies and Elling, 2015; Dropkin *et al.*, 1969). Resistance of the cruciferous vegetables to *H. trifolii* is post-infection especially associated with syncytial formation after penetration and after establishment of nutritional plant-nematode interactions (infection) (Agrios, 2005; Kim *et al.*, 1987). In these aspects, structural resistance mechanisms may be the depletion of syncytial cytoplasm at early stages of the nematode infection (shown at 10 DAI in the present study), but not syncytial sizes of no or little critically different characteristics between resistant and susceptible host responses.

In order to promote the plant growths, fertilizers are usually applied in fields at the optimum concentrations, which were also studied for the effectiveness in reducing the nematode growth and reproduction of *H. trifolii* in the present study. The soil contents of the major nutritional components such as total nitrogen (T–N), phosphate (P_2O_5) and potassium (K) increased proportionally with the increased fertilizer concentrations applied in soil, while other soil physico-chemical components were most significantly different among the fertilizer concentrations, suggesting the effects of different fertilizer concentrations on the nematode were exerted by the three major nutritional components. In the present study, the fertilizer concentration of 0.02% was most optimum in promoting plant growths such as root and shoot lengths, at which the nematode reproduction, fecundity and / or hatching rate were the minimum.

Heterodera schachtii and *Meloidogyne javanica* are effectively controlled by the soil amendment with poultry manure and application of chemical fertilizer (NPK) in soil (Esfahani, 2017). There are a variety of reasons for the population decreases of plant-parasitic nematodes that can be classified mainly by altering biotic and abiotic soil environments and promoting plant health-related defense mechanisms against the nematode (Akhtar and Malik, 2000). For example, supply of these nutrients alters microbial activity and diversity or affected by root extracts secreted from the plants affected by fertilizer treatments (Widmer *et al.*, 2002). However, nematode population changes by the fertilizer treatment are dependent on fertilizers and nematode: with inorganic N treatment; populations of *Criconemoides* and *Trichodorus* suppressed, but no significant population changes of *Tylenchorhynchys*: with urea treatment; populations of *Pratylenchus penetrans* and *Hoplolaimus* sp. inhibited, but no significant population changes of *Tylenchorhynchus* sp. (Rodriguez Kabana, 1985). It is presumed that reduced *H. trifolii* reproduction in soil with optimum fertilizer treatment was derived from the reduced penetration as in *Meloidogyne incognita* or reduced nematode growth as in *M. hapla* (Kim *et al.*, 2017). However, in the present study, the nematode sizes at 10 and 15 DAI treated with fertilizer 0.02% concentration were significantly smaller than the other concentrations, indicating the fertilizer treatment of 0.02% concentration may be effective in regarding the nematode growth in the root tissues. These aspects suggest that the reduced nematode reproduction at the optimum fertilizer treatment (0.02%) may be more derived from the attribution of fertilization to plant development than from the direct fertilizer

effect on soil environments. This may be additionally supported by the microbial activity and diversity in the soil may be derived from the root exudates released from the plants treated with the optimum fertilizer concentrations (Widmer *et al.*, 2002).

The plant growths (root length and shoot length) were significantly reduced by the nematode infection in the lowest fertilizer treatments (0.00% and 0.01%), respectively. This suggests plant damages caused by the nematode infection may be more severe in the low fertilizer concentrations than high concentrations. This is due to the more requirement of N for the production of plant defense hormones (Mur *et al*., 2017).

In conclusion, all of these aspects in the present study provide basic information useful for the development of the cultural control methods against *H. trifolii* such as the use of resistant plants and optimum fertilization.

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우리나라 십자화과 작물과 클로버씨스트선충의 생물학적 및 조직병리학적 상호작용

한가람

초록

Heterodera trifolii 는 2018 년 강원도 고랭지 배추 재배지에서 처음 분리되었다. 2011 년 보고된 *H. schachtii* 와 형태 및 증상이 유사하여 이 두 종이 혼합하여 피해를 입혔을 것으로 여겨진다. 따라서 본 연구는 *H. trifolii* 에 감염된 식물의 생물학적, 병리학적 특성을 파악하여 적절한 관리 방법을 도출하기 위해 실시하였다. 우리나라 십자화과 작물인 배추와 무, 5 가지 품종을 대상으로 선충 접종 28 일 후 확인한 증식률은 무보다 배추에서 현저히 높았다. 구체적으로, *H. trifolii* 의 cyst 형성율과 산란율 (cyst 당 알 수)은 배추가 무보다 높았고 부화율은 무와 배추에서 뚜렷한 차이를 나타내지 않았으나 초기 접종 선충 밀도 대비 최종 선충 밀도는 배추가 무보다 현저히 높았다. 형성된 cyst 의 수에 따라 순서대로 배추의 CR, 무의 송백, 금정을 선발하였고 2 일차, 5 일차 그리고 10 일차 침입률 조사 결과, 초기 침입률은 배추보다 무에서 높았지만 10 일차에 배추에서의 침입률은 빠른 속도로 증가하여 가장 높은 침입률을 나타내었다. 그러나 선충의 침입 이후 뿌리 내 선충의 생장은 품종 간 유의적인 차이가 없었다. 조직병리학적 반응을 통한 합포체의 발달을 조사한 결과 10 일차,

15 일차 모두 증식률이 가장 높았던 배추의 CR 품종에서 가장 큰 면적의 합포체를 형성하였다. 시기별 합포체의 면적 변화 조사에서 무에서의 선충 증식이 억제됨에 따라 합포체의 발달이 억제되는 것을 확인하여 합포체 발달이 선충의 생식력과 관련이 있음을 확인하였다. 식물의 발육을 촉진시키는 비료를 농도별로 처리하였을 때 식물의 지하부 생육이 가장 좋았던 비료의 농도에서 선충의 cyst 형성율과 수와 산란율이 가장 낮았고 산란율은 가장 높은 농도에서 유의적으로 높았다. 비료 처리 시 선충의 초기 침입률은 비료의 농도가 낮아질수록 높게 나타났지만 최고 또는 그 다음의 비료 농도에서는 초기 침입률이 낮을 뿐만 침입 이후 선충의 정상적인 생장을 억제하여, 최종적인 cyst 형성율이 낮아지는 결과를 초래하였다. 이러한 연구결과에서 *H. trifolii* 의 증식은 합포체의 발달과 관련이 있고, 비료의 농도에 따른 선충의 증식, 침입, 성숙에 대한 처리효과가 확인됨에 따라 *H. trifolii* 의 방제전략을 도출하는데 유효한 정보를 제공할 것으로 생각된다.

주요어: *Heterodera trifolii*, 십자화과 작물, 비료

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